Downregulated inhibitor of growth 3 (ING3) expression during colorectal carcinogenesis

Wen-feng Gou, Hong-zhi Sun, Shuang Zhao, Zhe-feng Niu, Xiao-Yun Mao[*], Yasuo Takano[+] & Hua-chuan Zheng

Cancer Research Center, The First Affiliated Hospital of Liaoning Medical University, JinZhou, PR China; [*]Department of Surgical Oncology, The First Affiliated Hospital of China Medical University, Shenyang, PR China & [+]Clinical Research Institute, Kanagawa Cancer Center, Yokohama, Japan

Background & objectives: ING3 (inhibitor of growth protein 3) overexpression decreased S-phase cell population and colony-forming efficiency, and induced apoptosis at a p53-mediated manner. The aim of this study was to investigate the clinicopathological and prognostic significance of ING3 expression in colorectal carcinogenesis and subsequent progression.

Methods: ING3 expression was examined by immunohistochemistry on tissue microarray containing colorectal non-neoplastic mucosa (NNM), adenoma and adenocarcinoma. Colorectal carcinoma tissue and cell lines were studied for ING3 expression by Western blot or RT-PCR.

Results: ING3 mRNA was differentially expressed in Colo201, Colo205, DLD-1, HCT-15, HCT-116, HT-29, KM-12, SW480, SW620 and WiDr cells. Carcinomas showed significantly lower *ING3* expression than matched NNM at mRNA level ($P<0.05$), but not at protein level. Immunohistochemically, ING3 expression was significantly decreased from NNM, adenoma to adenocarcinoma ($P<0.05$). ING3 expression was not correlated with age, sex, tumour size, depth of invasion, lymphatic or venous invasion, lymph node metastasis, tumour- node- metastasis staging or differentiation. Kaplan-Meier analysis indicated that ING3 protein expression was not associated the prognosis of the patients with colorectal carcinoma ($P<0.05$).

Interpretation & conclusions: Our study showed that downregulated ING3 expression might play an important role in colorectal adenoma-adenocarcinoma sequence. Further studies are required to understand the mechanism.

Malignant transformation is genetically a complex process, featuring frequent genetic and epigenetic alterations activating oncogenes and inactivating tumour suppressor genes (TSG)[1]. Among these, chromosomal deletion, mutation or hypermethylation which can lead to loss (Class I) or inactivation (Class II) of TSG causing immortality of cancer cells, has provided clues to the identification of genes critical for initiation, promotion and development of tumours, such as the inhibitor of growth (ING) family[2].

ING proteins consist of five members with various isoforms and contain a highly conserved plant homeodomain (PHD), a Cys_4-His-Cys_3 form of zinc finger interacting directly with histone H3, and a nuclear localization sequence (NLS). ING proteins could function as receptors and transducers of stress-activated phosphoinositides, inhibit angiogenesis, promote cellular senescence, or be involved in various biological processes, including the apoptosis, DNA repair, cell cycle checkpoints, histone methylation and acetylation, regulators of transcription by protein-protein or protein-DNA interaction[3-5]. The human *ING3* gene originally cloned by the genetic suppressor element methodology, is mapped to 7q31.3, consists of 12 exons, and encodes a 46.8 kDa protein that modulates p53-mediated transcription, cell cycle control, and apoptosis[6]. ING3 is predominantly present in the NuA4 histone acetyltransferase (HAT) multisubunit complex and required for the histone acetyltransferase activity of Tip60[7,8]. ING3 overexpression decreased S-phase cell population of cells and colony - forming efficiency, and induced apoptosis in RKO cells at a p53-mediated manner. Further study indicates that ING3 activates p53-transactivated promoters of p21/waf1 and bax[6]. ING3 promotes UV-induced apoptosis of melanoma cells by increasing the cleavage of Bid and caspases-8, -9, and -3, and upregulating Fas expression[9]. Furthermore, it was found that ING3 underwent degradation via its interaction with subunits of E3 ligase Skp1-Cullin-F-box protein complex (SCF complex) in the ubiquitin-proteasome pathway, which gives another explanation for ING3 downregulation[10].

ING3 is ubiquitously expressed in normal human tissues, including spleen, testis, skeletal muscle, heart and oral mucosa[6]. In malignant melanoma, nuclear ING3 expression was remarkably reduced compared with dysplastic nevi and significantly correlated with a poor prognosis of melanoma as an independent factor[11]. Gunduz *et al*[12] reported that loss of heterozygosity resulted in reduced ING3 expression in human head and neck squamous cell carcinomas. Borkosky *et al*[13]. found that SSLOH of ING3MS (ING3 locus) was also high in solid type tumours of ameloblastoma. These findings have suggested that decreased ING3 expression may be associated with the tumorigenesis and subsequent development of malignancies.

Colorectal cancer is one of the most common cancers in the world, accounting for nearly 10 per cent of new cases of all cancer[14]. Japan has experienced a marked increase in the incidence of colorectal cancer, and has recently been listed in the group of countries with the world's highest incidence rates[15]. Pathological and genetic observations demonstrated that colorectal adenoma precedes the majority of colorectal adenocarcinoma and could undergo malignant transformation into adenocarcinoma. However, the molecular mechanisms underlying this colorectal carcinogenesis are still poorly understood. In this study, *ING3* expression was examined in colorectal carcinoma, adenoma, non-neoplastic mucosa and carcinoma cell lines, and compared with the clinicopathological parameters of carcinomas, as well as prognosis to explore the clinicopathological significance of ING3 expression in the development of colorectal carcinoma.

Material & Methods

Cell culture: Colorectal carcinoma cell lines (kindly donated by Prof. Sugiyama, Department of Gastroenterology, Graduate School of Medical and Pharmaceutical Sciences, University of Toyama, Japan, and Prof. Miyagi, Clinical Research Institute, Kanagawa Cancer Center, Japan). were maintained in Roswell Park Memorial Institute (RPMI) 1640 (Colo201, Colo205, DLD-1, HCT-15, HCT-116, HT-29, KM-12, SW480 and SW620) and Dulbecco's Modified Eagle's Medium (DMEM) (WiDr) medium supplemented with 10 per cent foetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5 per cent CO_2 at 37°C.

Subjects: Colorectal carcinomas (CRC, n=319) and adjacent matched non-neoplastic mucosa (NNM) were blindly and randomly collected from the surgical resection in Kouseiren Takaoka Hospital (Japan) between 1993 and 2002, and adenoma (n=96) from endoscopic biopsy in the Affiliated Hospital, University of Toyama (Japan). The patients with CRC were 179 men and 140 women (18-90, mean= 68.6 ± 7.9 yr). The cases with malignant transformation in colorectal adenoma were excluded from the present study during tissue microarray making process. Among them, 126 cases were accompanied with lymph node metastasis. Twenty cases of CRC and paired NNM were collected from the First Affiliated Hospital of China Medical University, Shenyang (China) and frozen at -80°C until RNA or protein extraction by homogenization. None of the patients underwent chemotherapy, radiotherapy or adjuvant before surgery. They all provided the written consent for use of tumour tissue for clinical research and the University Ethical Committee of Liaoning Medical University, Jinzhou, China approved the research

protocol. We followed up 319 patients by consulting their case documents or by telephone from October 10, 1997 to February 8, 2008. The loss of follow up was 14.1 per cent (45/319) in the present study and 111 (34.8%) patients died from colorectal carcinoma during the period.

Pathology and tissue microarray (TMA): All tissues (n=734) were fixed in 10 per cent neutral formalin, embedded in paraffin and cut into 4 µm thick sections. These sections were stained by hematoxylin- and-eosin (HE) to confirm the histological characteristics. According the depth of invasion, 29 cases of early carcinoma and 290 cases of advanced carcinoma were included in the study. Based on WHO classification[16], there were 162 cases of well-differentiated, 128 cases of moderately-differentiated and 29 cases of poorly-differentiated carcinoma. The staging for each colorectal carcinoma was evaluated according to the Union Internationale Contre le Cancer (UICC) system[17]. Elastic-van Gieson (EvG) staining and D2-40 immunostaining were employed to determine the venous and lymphatic invasion, respectively. The tumour size was grossly determined.

Representative areas of solid tumours were identified in HE-stained sections of the selected tumour cases and a four mm-in-diameter tissue core per donor block was punched out and transferred to a recipient block with a maximum of 24 cores using a Tissue Microarrayer (AZUMAYA KIN-1, Japan).

RT-PCR: Total RNA was extracted from colorectal carcinoma cell or tissues using QIAGEN RNeasy mini kit (QIAGEN, Germany). Two micrograms of total RNA was subjected to cDNA synthesis using the Avian Myeloblastosis virus (AMV) transcriptase and random primer (Takara, Japan). Oligonucleotide primers were Forward: 5'- CTTCACGGAAATGCG -3' and Backward 5'- CTCTTCCCTCCACTCA -3' for *ING3* (118 bp). The primers for an internal control, glycereldehyde-3-phosphate dehydrogenase (*GAPDH*), were forward: 5'- CAATGACCCC TTCATTGACC -3' and reverse: 5'- TGGAAGATGGTGATGGGATT -3' (135 bp). General PCR amplification of cDNA was performed in 25 µl mixtures using Pfu (Stratagene, USA) and the amplicons were electrophoresized in 2% agarose gel for 30 min. These primers were synthesized by Takara Biotech company (Dalian, PR China).Real-time PCR was performed according to the protocol of SYBR Premix Ex Taq™ II kit (Takara, Japan). The expression level of ING3 was expressed as $2^{-\Delta Ct}$, where $\Delta Ct = Ct (REIC) - Ct(GAPDH)$. Additionally,

the expression level of the normal mucosa tissue was considered as "1".

Western blot: Protein assay was performed using Biorad protein assay kit (Biorad, USA). The denatured protein was separated on an sodium dodecyl sulphate (SDS)-polyacrylamide gel and transferred to Hybond membrane (Amersham, Germany), which was then blocked overnight in 5 per cent milk in Tris buffered saline, with Tween 20 (TBST). For immunobloting, the membrane was incubated for 1 h with rabbit anti-ING3 antibody (Protein Tech Group Inc, USA; 1: 500) or mouse anti-β-actin (Santa Cruz, USA). Then, it was rinsed by TBST and incubated with anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (DAKO, USA, 1:1000) for 1h. Bands were visualized by ECL-Plus detection reagents (Santa Cruz).

Immunohistochemistry: The immunohistochemistry was performed according to the procedures recommended by Kumada *et al*[18]. The rabbit anti-ING3 (Protein Tech Group Inc, USA; 1 : 100) was employed as the primary antibody. Then, anti-rabbit Envison-PO (DAKO; 1:100) antibody was subjected to incubation as the secondary antibody. Binding sites were visualized with 3, 3'-diaminobenzidine, followed by the counterstaining with Mayer's hematoxylin. Immunoreactivity for ING3 was localized in the cytoplasm. One hundred cells were randomly selected and counted from five representative fields of each section. The positive percentage of counted cells was graded semi-quantitatively according to a four-tier scoring system: negative (-), 0~5 per cent; weakly positive (+), 6~25 per cent; moderately positive (++), 26~50 per cent; and strongly positive (+++), 51~100 per cent.

Statistical analysis: Statistical evaluation was performed using Spearman correlation test to analyze the rank data about the relationship between ING3 immunostaining and colorectal carcinogenesis or carcinoma aggressive parameters. The student t test was used to compare the means of different groups. Kaplan-Meier survival plots were generated and comparisons were made with the log-rank test. Cox's proportional hazards model was employed for multivariate analysis. *P*<0.05 was considered significant and SPSS 10.0 software (SPSS Inc., USA) was employed to analyze all data.

Results

ING3 expression in colorectal carcinoma cells and tissue: *ING3* mRNA was strongly expressed in Colo 201, Colo 205, DLD-1, HCT-116, HCT-15 and HT29,

while its comparatively week expression was observed in KM-12, SW480, SW620 and WiDr. Among frozen cases, *ING3* mRNA expression was significantly lower in colorectal carcinoma than paired NNM by real-time PCR ($P<0.05$). ING3 protein expression was detectable in all samples, but there was no difference in ING3 expression between carcinoma and matched NNM.

Expression of ING3 distributed to the cytoplasm of colorectal NNM, adenoma, carcinoma and infiltrated inflammatory cells (Fig. 1). Overall, ING3 expression was detected in 99.3 per cent of NNMs (317/319), 83 out of 96 adenomas (86.5%), and 192 out of total 319 carcinomas (60.2%). Combined with the intensity and frequencies, ING3 expression showed remarkable decrease from colorectal NNM to adenocarcinoma through adenoma ($P<0.001$, Table I).

ING3 expression and its correlation with clinicopathological parameters of colorectal carcinomas: As Table II summarized, ING3 expression was not correlated with any aggressive behaviours of colorectal cancer, including age, sex, tumour size, depth of invasion, lymphatic or venous invasion, or lymph node metastasis, TNM staging, or differentiation. Follow up information was available on 274 patients with colorectal carcinoma and period ranged from 0.9 months to 12.1 years (median=66.3 months). Kaplan-Meier analysis indicated that there was no significant difference between cumulative survival rate of the patients with weak, moderate or strong

ING3 expression and without its expression (Fig. 2). Cox's analysis demonstrated that lymphatic and venous invasion, lymph node metastasis, and UICC staging were independent prognostic factors for overall colorectal carcinomas ($P<0.05$, Table III).

Discussion

Many studies have shown evidence for alteration or downregulation of ING genes in malignancies[2-4]. *ING2* was shown to be downregulated at both the transcriptional and post-translational levels and was closely associated with a poor survival rate in hepatocellular carcinoma[19]. Another report demonstrated low levels of *p33/ING1* and *p47/ING1* mRNAs in colorectal cancer tissues, compared to normal tissues, and positively correlated with Duke's staging[20]. Dramatically reduced expression of p33 ING1 and *ING4* mRNAs was shown in gastric carcinoma tissues[21-23]. We have earlier reported upregulated *ING5* mRNA expression and the nuclear to cytoplasmic shift of ING5 protein during colorectal carcinogenesis[24,25]. Chromosomal deletion and/or decrease of *ING4* expression has also been reported in glioma[26] and breast cancer[27]. Taken together, these findings suggest that abnormal expression or alteration of *ING* genes contributes to the pathogenesis of malignant cancers.

In the present study, ING3 expression was studied during colorectal carcinogenesis and cytoplasmic ING3 was present in colorectal carcinoma, paired NNM, and
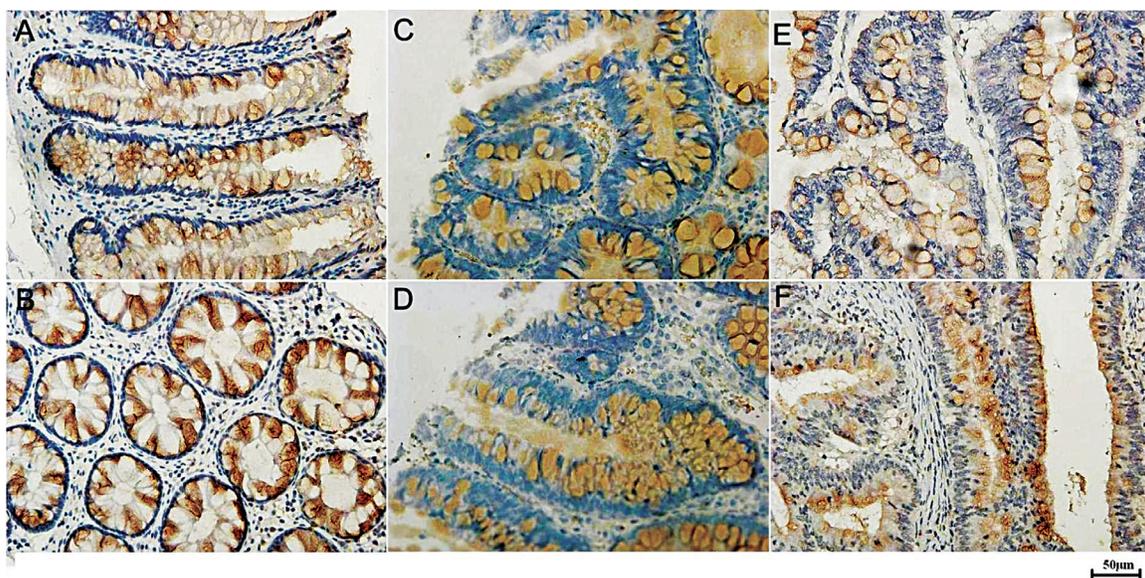

Fig. 1. Immunostaining examination of ING3 protein in colorectal lesions. ING3 immunoreactivity was detectable in colorectal mucosa **(A, B)**, adenoma **(C, D)**, carcinoma **(E, F)** and infiltrating inflammatory cells **(A, F)**. (X 200 magnification).

Table I. *ING3* expression in colorectal non-neoplastic, adenomas and carcinoma						
Groups	n	*ING3* expression				
		−	+	++	+++	PR (%)
Non-neoplastic mucosa	319	2	62	219	36	99.3
Adenoma	96	13	19	53	11	86.5*
Carcinoma	319	127	86	83	23	60.2*

ING3, inhibitor of growth 3; PR, positive rate; NNM. *$P<0.001$ compared with non-neoplastic mucosa; (-), negative; (+), weekly positive; (++), moderately positive; (+++), strongly positive

Table II. Relationship between *ING3* expression and clinicopathological features in colorectal carcinoma								
Clinicopathological features	n	*ING3* expression						
		−	+	++	+++	PR (%)	R	*P* value
Age(yr)							0.045	0.429
<65	125	53	36	23	13	57.6		
≥65	192	73	50	59	10	62.0		
Sex							-0.026	0.651
Male	179	71	46	47	15	60.3		
Female	140	56	40	36	8	60.0		
Tumour size (cm)							-0.020	0.729
≤5	211	82	59	55	15	61.1		
>5	107	45	27	27	8	57.9		
Differentiation degree							0.054	0.340
Well	162	72	37	39	14	55.6		
Moderately-poorly	157	55	49	44	9	65.0		
Depth of invasion							-0.024	0.667
T_{is}-T_1	26	10	6	8	2	61.5		
T_2-T_4	289	116	80	74	19	59.9		
Lymphatic invasion							0.030	0.594
-	223	94	55	59	15	57.8		
+	87	31	29	20	7	64.4		
Veinous invasion							-0.017	0.773
-	264	108	65	71	20	59.1		
+	46	16	19	9	2	65.2		
Lymph node metastasis							-0.029	0.610
-	188	72	53	50	13	61.7		
+	126	53	32	33	8	57.9		
TNM staging							-0.085	0.171
I-II	68	21	21	20	6	69.1		
II-IV	195	82	49	49	15	57.9		

R, spearman's correlation coefficient; PR, positive rate; T_{is}, carcinoma *in situ*; T_1, involvement of the lamina propria and submucosa; T_2, muscularis propria and subserosa; T_3, serosa; T_4, invasion through the serosa
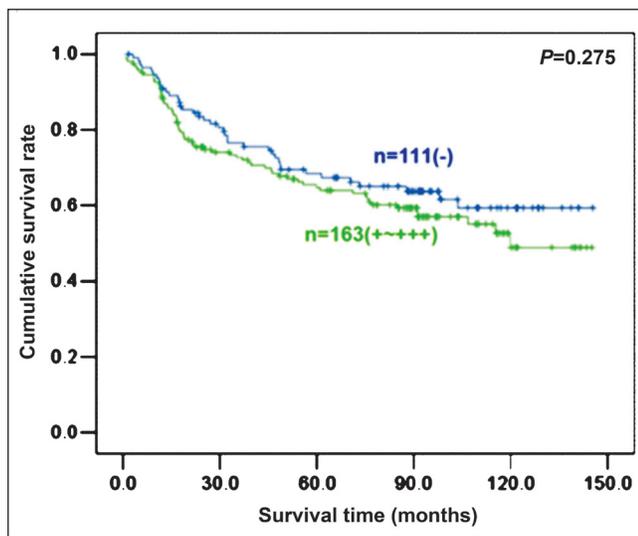
Fig. 2. Correlation between ING3 status and prognosis of the colorectal carcinoma patients. Kaplan-Meier curves for cumulative survival rate of patients with colorectal carcinomas according to ING3 expression status.

adenoma. ING3 expression was significantly reduced from NNM, adenoma to adenocarcinoma. In addition, *ING3* mRNA content was lower in carcinoma than NNM. These findings suggested that downregulated ING3 expression might be involved in the colorectal carcinogenesis as an early event. In contrast, there was no difference in ING3 protein content between NNM and cancer tissue. The paradoxical phenomenon might be attributable to the mixture of inflammatory or stromal cells, the antibody specificity or the complexity of protein synthesis and degradation. Wang *et al*[11] showed decreased ING3 expression in malignant melanoma in comparison with dysplastic nevi and Gunduz *et al*[12,28] found allelic loss and reduced expression of the ING3 in human head and neck cancers, in line with our findings. Because ING3, a tumour suppressor, plays an important role in the chromosomal remodelling, apoptotic induction and proliferative inhibition and its downregulation by ubiquitin-proteasome pathway might contribute to its functional dysregulation, its decreased expression may lead to disruption of these processes, thereby contributing to tumorigenesis[2-4,8-10]. Interestingly, we found no nuclear expression pattern of ING3 although the same antibody was employed by Wang *et al*[11]. The distinct results might be due to different immunostaining approaches and tissue specificity of ING3 immunoreactivity.

To clarify the *in situ* expression pattern and the clinicopathological significance of ING3 protein,

immunohistochemistry was performed on tissue microarray (TMA) of colorectal lesions. We found no significant relationship between ING3 expression and aggressive pathological features of CRC, including depth of invasion, lymphatic and venous invasion, and lymph node metastasis consistent with other reports[11,12,28]. In addition, there was no correlation of ING3 expression with survival time of the patients with CRC. However, the reduced nuclear ING3 expression was reported to significantly correlate with a poorer disease-specific 5-year survival of patients with primary melanoma, particularly as an independent prognostic factor for high-risk melanomas[11]. Gunduz *et al*[28] documented that ING3 expression might be employed to indicate a favourable prognosis of head and neck squamous cancer as an independent factor. Our further analysis suggested that lymphatic and venous invasion, lymph node metastasis, and UICC staging were independent prognostic factors for CRC.

In summary, our study indicates that downregulated ING3 expression may have an impact on the malignant transformation of colorectal epithelial cells and could be considered as a biomarker for colorectal carcinogenesis. Understanding of the biological functions of the ING3 proteins in malignancies requires further investigation.

Table III. Multivariate analysis of clinical variables for colorectal carcinoma

Clinicopathological parametres	Relative risk (95%CI)	P value
Age ($\geq$65 yr)	1.529 (0.965-2.422)	0.071
Sex (female)	1.047 (0.671-1.633)	0.841
Tumour size ($\geq$5 cm)	0.944 (0.602-1.481)	0.802
Depth of invasion (T_{2-4})	2.104 (0.274-16.144)	0.474
Lymphatic invasion (+)	1.710 (1.063-2.752)	0.027
Venous invasion (+)	2.116 (1.211-3.700)	0.009
Lymph node metastasis (+)	2.665 (1.653-4.296)	<0.001
UICC staging (II-IV)	2.194 (1.086-4.435)	0.029
Differentiation degree (moderately-poorly)	1.029 (0.650-1.630)	0.902
ING3 expression (+~+++)	1.188 (0.765-1.845)	0.443

CI, confidence interval; UICC, Union Internationale Contre le Cancer

Acknowledgment

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Reprint requests: Prof. Hua-chuan Zheng, Cancer Research Center, The First Affiliated Hospital of Liaoning
Medical University, JinZhou 121 001, PR China
e-mail: zheng_huachuan@ hotmail. com